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Serum Glutamate Levels Correlate with Gleason Score and Glutamate Blockade Decreases Proliferation, Migration, and Invasion and Induces Apoptosis in Prostate Cancer Cells

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Abstract

Purpose—During glutaminolysis, glutamine is catabolized to glutamate and incorporated into citric acid cycle and lipogenesis. Serum glutamate levels were measured in patients with primary prostate cancer (PCa) or metastatic castrate-resistant PCa (mCRPCa) to establish clinical relevance. The effect of glutamate-deprivation or blockade by metabotropic glutamate receptor 1 (GRM1)-antagonists was investigated on PCa cells' growth, migration, and invasion to establish biological relevance.

Experimental Design—Serum glutamate levels were measured in normal men (n = 60) and patients with primary PCa (n = 197) or mCRPCa (n = 109). GRM1 expression in prostatic tissues was examined using immunohistochemistry (IHC). Cell growth, migration, and invasion were

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determined using cell cytotoxicity and modified Boyden chamber assays, respectively. Apoptosis was detected using immunoblotting against cleaved caspases, PARP and γ -H2AX.

Results—Univariate and multivariate analyses demonstrated significantly higher serum glutamate levels in Gleason score 8 than in the Gleason score 7 and in African Americans than in the Caucasian Americans. African Americans with mCRPCa significantly higher serum glutamate levels than those with primary PCa or benign prostate. However, in Caucasian Americans, serum glutamate levels were similar in normal research subjects and patients with mCRPC. IHC demonstrated weak or no expression of GRM1 in luminal acinar epithelial cells of normal or hyperplastic glands, but high expression in primary or metastatic PCa tissues. Glutamate deprivation or blockade decreased PCa cells' proliferation, migration, and invasion and led to apoptotic cell death.

Conclusions—Glutamate expression is mechanistically associated with and may provide a biomarker of PCa aggressiveness.

Keywords

Glutamate; GRM1; Biomarker; Prostate Cancer; Metastasis; Castrate-resistant

Prostate cancer (PCa) is estimated to account for 29% of all new cancers and is the second leading cause of cancer-related death in men in the United States in 2012 (1). Age, African-American race, and family history are unequivocal risk factors for PCa (2). Focusing on early detection of PCa has the capacity to decrease PCa mortality. Serum prostate-specific antigen (PSA) is used for PCa early detection, but its use is controversial because of its sensitivity and specificity is limited and it does not provide reliable prognostic information. These limitations have led investigators to consider new approaches, using systems biology, to identify biomarkers of PCa aggressiveness. This goal could be reached by defining important interacting cellular networks and/or metabolic pathways that reflect tissue- and disease-specific phenotypes.

Metabolomics has emerged recently as a promising method for PCa detection (3), one that may supplement, or even replace current PSA testing. Metabolomics profiling of PCa offers the potential for early detection and discrimination of clinically aggressive tumors from indolent and non-aggressive diseases. Metabolic changes are affected by genetic and epigenetic changes and a long list of environmental factors, which include diurnal variation, dietary habits, and lifestyle. The prostate is a unique target for metabolomics analysis both under normal conditions and upon neoplastic transformation. Extremely high amounts of citrate are produced and secreted by the prostate that reflects the unique state of anabolic and catabolic processes of secretory luminal cells. Citrate metabolism differs in PCa compared to benign prostatic hyperplasia (BPH) (4).

Glutamate is a non-essential amino acid and an excitatory neurotransmitter that is involved in neuroembryonic growth and development by regulating proliferation, survival, migration, and invasion of neuronal progenitors and immature neurons (5). A prominent consequence of signaling in rapidly proliferating cancer cells is increased nutrient uptake. Glucose and glutamine are the two most abundant nutrients in mammals. During glycolysis and glutaminolysis, glucose is converted to lactate and glutamine to glutamate, respectively (6). In addition to being a precursor of other amino acids and nucleotides, glutamate metabolism is closely linked to ureagenesis, glutathione synthesis, tricarboxylic acid cycle (TCA), amino acid transferase, GABA synthesis, lipogenesis, and ATP production (6). More than 80 years ago, Otto Warburg demonstrated aerobic glycolysis in tumor cells by their high rate of wasteful glucose consumption, increased lactate production, and bioenergetics demand despite accessibility to oxygen (7). This shift from mitochondrial ATP production was later

proved to be a hallmark of aggressive tumors and rapidly proliferating non-transformed cells, such as lymphocytes, thymocytes, and erythrocytes (8). In the PC-3 PCa cell line, c-Myc oncogenic transcription increases mitochondrial glutaminase expression that leads to enhanced glutaminolytic activity, catabolic conversion of glutamine to glutamate and glutamine addiction (9). Taylor *et al* demonstrated elevated levels of glutamate in 12 PCa compared with 16 BPH tissues and increased levels of additional metabolites in the urea cycle using high throughput quantitative humoral response profiling (10). Glutamate levels were higher in the majority of PCa patients than in BPH tissues in a follow-up study by the same group (11).

Glutamate in secreted form is a phylogenetically conserved cell signaling molecule in addition to its intracellular activities in bioenergetics, biosynthetic pathways, maintaining amino acids and nucleotide pool, and metabolism (12). The glutamatergic system comprises the glutamate, glutamate receptors (GluRs), and glutamate transporters. The GluRs are divided into two different categories: the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs) (13). The iGluRs are gated ion channels with excitatory action (13). The mGluRs promote cellular signaling pathways via seven transmembrane domain G-protein coupled receptors (GPCRs) and are subdivided into three groups and eight subtypes: group I (mGluR1 and mGluR5), II (mGluR2 and mGluR3), and III (mGluR4, mGluR6, mGluR7, and mGluR8) based on their pharmacology, sequence homology, response to agonists, and downstream signaling. mGluRs are expressed in a variety of non-neuronal cell types, such as hepatocytes, melanocytes, keratinocytes, myocardial cells, pancreatic cells, and embryonic stem cells (14, 15). Recently, several studies have indicated the involvement of mGluRs in tumorigenesis. mGluRs are highly overexpressed in the nervous system, so initial discoveries of mGluRs in human tumors were reported in neuro-glial derived tumors such as gliomas, neuroblastoma, and medulloblastoma (14, 15). Glioma cells secreting high amounts of glutamate have higher growth rates than their isogenic parental cells (16). Glutamate antagonists decreased proliferation of a variety of cancer cells which include colon adenocarcinoma, melanoma, lung carcinoma, thyroid carcinoma, breast carcinoma, astrocytoma, neuroblastoma, and rhabdomyosarcoma (17). The first proof for the involvement of mGluRs in non-neuronal tumorigenesis was obtained by Chen *et al* (18, 19). A transgenic mouse line overexpressing mGluR1 (also known as GRM1) in melanocytes was predisposed to spontaneous melanoma development with 100% penetrance, short latency, and high metastatic potential (18,19). In PCa, mGluR1-5 mRNA expression was reported in PC-3 and LNCaP cells and mGluR6 and 8 were expressed only in LNCaP cells. DHT induced mGluR7 expression and inhibited mGluR8 expression in LNCaP cells (20). The clinical significance or biological relevance of glutamate in PCa has not been investigated.

In this study, we examined the association between serum glutamate levels and PCa aggressiveness to establish clinical relevance. To determine the biological relevance, the effect of glutamate deprivation or blockade was investigated on PCa cells' growth, migration, and invasion.

Materials and Methods

Patients and samples

Primary PCa cases investigated in this study refer to newly diagnosed, untreated, and clinically localized PCa. Normal research subjects in this study are defined as men with no evidence of PCa. Serum samples used to evaluate glutamate levels in normal individuals and men with primary tumor or mCRPCa, were obtained from the biospecimen core facilities at the Louisiana Cancer Research Consortium (LCRC) affiliated to Tulane Medical School and School of Medicine, Louisiana State University Health Sciences Center (LSUHSC) and the

tumor bank at the University of Washington Medical Center (UWMC) after informed consent and without any personal identifiers. Briefly, peripheral blood samples were collected by phlebotomy from consenting normal adult males or patients with primary or mCRPCa in the Urology Oncology clinics at the UWMC and LCRC-affiliated hospitals. All of the controls came from patients who presented at the University of Washington's annual free PSA screening week. All patients were above 40 years of age and all stated that they did not have a history of prostate cancer. PSA values were below 4.0 ng/mL. Blood samples from UWMC were processed by centrifugation and serum samples were stored in aliquots at -80°C within 4 hours of draw. Overall, all normal serum samples, 39% of primary PCa, and 69% of mCRPCa samples were obtained from the UWMC. The remainder of serum samples was processed similarly and obtained from the biospecimen core facilities at LCRC. Serum samples were stored until used in the research study; no freeze-thaw cycles were incurred. We did not acquire fasting or post meal status on any of the research subjects. PCa aggressiveness was defined using Gleason score, clinical stage, and PSA at diagnosis as: (a) high aggressive (Gleason sum ≥ 8 , or PSA >20 ng/ml, or Gleason sum ≥ 7 , and stage cT3-cT4); (b) low aggressive (Gleason sum <7 and stage cT1-cT2 and PSA <10 ng/ml); or (3) intermediate aggressive (all other cases) (21). The PCa tissue specimens used for IHC were obtained from Accumax Array (Accurate Chemical & Scientific Corp, Westbury, NY).

Ethical considerations

Ethical approval was obtained from the Institutional Review Boards at University of Washington Medical Center, Seattle, Washington, Tulane Cancer Center, Tulane University School of Medicine, New Orleans, Louisiana, LSUHSC, School of Medicine, New Orleans, LA, and Roswell Park Cancer Institute, Buffalo, New York, NY.

Determination of serum glutamate and creatinine levels

Serum glutamate levels were measured using BioVision's glutamate assay kit (#K629-100, Mountain View, CA) that provides sensitive detection method for glutamate in a variety of biological samples and fluids including serum, urine, cells, and tissue extracts. The amount of glutamate is quantitated by measuring optical density at $\lambda = 450$ nm in a microplate reader. Background reading was corrected by subtracting the value from the zero glutamate control from all samples readings. Serum creatinine concentration was measured using the BioVision creatinine assay kit (#K625-100) and the manufacturer's instructions. After plotting the independent glutamate and creatinine standard curves, glutamate and creatinine concentrations of the serum samples were calculated using the formula $C = S_a/S_v$ nmol/ μL , or mM, where S_a is the sample amount of unknown (in nmol) from standard curve and S_v is the sample volume (μL) added to each well.

The minimum detectable amounts for serum glutamate or creatinine were two nmol/mL. The standard curve reproducibility was demonstrated in five independent assays. The intra-assay variance was evaluated in ten replicates for three serum samples of known concentrations on one plate. The inter-assay precision was tested in three independent assays for three control samples in triplicates. Stability of serum glutamate was determined by incubating aliquots of serum samples of known glutamate concentrations at -20 or -70°C for 3, 6, 12, 18, 24, and 36 months.

Immunohistochemical analysis

The ABC detection system (Vector Laboratories Inc.) was used for immunostaining as described previously (22). Affinity purified, polyclonal rabbit antihuman-GRM1 antibody (at 1:250, Sigma) was used as the primary antibody. The incubation time for primary antibody was overnight at 4°C . The slides were counterstained with hematoxylin and mounted for examination. The negative control slides were treated in an identical manner

except that the primary antibodies were omitted. Descriptive examinations of cell type-specific anti-GRM1 immunoreactivity in various tissue constituents, normal, hyperplastic, and neoplastic glands in the sections were completed by the study pathologists.

Cell Culture, reagents, and western blot analysis

Androgen-independent (PC-3, DU145, MDA-PCa2b, VCaP, 22Rv1) and androgen-sensitive (LNCaP) PCa cell lines were purchased from American Type Culture Collection (Manassas, VA) and maintained in their individually specified medium (23). E006AA cell line was maintained in DMEM supplemented with 10% FBS (23). Non-competitive GRM1 antagonists (Riluzole and BAY36-7620) were purchased from Tocris (Ellisville, MO) (24). Customized glutamine- and glutamate-free RPMI-1640 or DMEM, dialyzed FBS, and GlutaMax™-I (a dipeptide, L-alanine-L-glutamine) were purchased from Life Technologies (Grand Island, NY). GlutaMAX was used as a substitute for glutamine in cell growth, which cannot be degraded or converted into glutamate. Cells were cultured in complete media up to 75% confluency, washed with cold PBS, and whole cell lysates were extracted as described previously (23). GRM1 was detected using a rabbit polyclonal antibody against human GRM1 (Cat # 19955-1-AP at 1:1000; Proteintech Group Inc, Chicago, IL) using ECL detection system (GE Healthcare Biosciences, Pittsburgh, PA). The anti-GRM1 specificity was examined by probing the duplicate membrane with primary antibody that was pre-incubated with GRM1-specific blocking peptide (Proteintech Group Inc, Chicago, IL) at 5 µg/ml for 2 hours with rotation at room temperature. Detection of apoptosis markers was performed using immunoblotting. Cells were cultured up to 50% confluency in complete culture media in the presence or absence of DMSO or riluzole at 0, 10, 25, or 50 µM for 4 or 6 days and whole cell lysates were prepared. Clarified protein samples (35 µg) were subjected to SDS-PAGE under reducing conditions. Western analyses were carried out using monoclonal antibodies against cleaved caspases-9, -7, and -3 and poly (ADP-ribose) polymerase (PARP) provided in an Apoptosis Sampler Kit (Cell Signaling Technology) and phosphorylated histone H2AX^{Ser 139} (γ-H2AX). Anti-GAPDH (at 1:5000; Cell Signaling Technology, Boston, MA) was used for loading controls. Each experiment was performed in duplicate, and the assays were repeated three times.

Cell proliferation assays

PCa cells growth was examined in the presence of GRM1 (mGluR1) antagonists, riluzole and BAY36-7620. Riluzole functions in part by blocking GRM1 signaling and preventing intracellular glutamate release. BAY 36-7620 is a specific non-competitive antagonist of GRM1 that binds the transmembrane domain of the receptor and stabilizes its inactive transformation (24). Cells were seeded at 500 (PC-3, DU145, LNCaP) per 200 µl/well and in 24 replicates in 96-well plates in complete medium. After 3 days culture medium was refreshed and supplemented with DMSO, riluzole, or BAY36-7620 at 0, 1, 10, 25, or 50 µM. Culture plates were incubated at 37°C for 2, 4, or 6 days and the media were refreshed every 48 h. Cell proliferation was measured using The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

PCa cell growth under glutamine- and glutamate-free condition was examined in an independent set of 96-well plates and at cell densities described above. Cells were incubated in customized glutamine- and glutamate-free culture media supplemented with 10% dialyzed FBS and 2 mM GlutaMAX™ for 3, 5, or 7 days and the media were refreshed every 48 h. After the indicated incubation times, cell proliferation was measured as described above and compared with their parallel isogenic cell lines cultured simultaneously but in their complete growth medium.

Cell migration and invasion assays

Cell migration and invasion assays were performed using 8- μ m transwell filters (Costar, Corning, NY) with the modifications described previously (25). For the invasion assay, the upper compartment was coated with 50 μ g Matrigel (BD Biosciences, San Jose, CA) to form a matrix barrier. A suspension of cells (5×10^4 for PC-3 or 2×10^4 for DU145) in basal medium containing 0.1% BSA was added to the upper compartment. The lower compartment was filled with 400 μ l basal medium containing 10% FBS as chemoattractant. Riluzole or BAY36-7620 was included at equimolar concentrations (0 to 50 μ M) to upper and lower compartments. After 48 h for PC-3 or 24 h for DU145, the non-migratory cells on the upper surface were removed using a cotton swab and the cells on the lower surface were fixed and stained with Diff-Quick solution (Dade Behring, Deerfield, Illinois). Migrated or invaded cells in transwell filters were counted from ten randomly selected fields in at least four independent wells. The experiment was repeated three times independently.

Statistical design and data analysis

All analyses were performed using SAS 9.3 (SAS Institute, Cary NC) with a nominal significance level of 0.05. The serum samples were stratified by clinical status of the research subjects (normal, primary PCa, or mCRPCa) with patient demographics (e.g., age, race) reported as mean and standard deviation for continuous variables, and as frequencies and relative frequencies for categorical variable (Supplementary Table S1 and S2). Patients with primary PCa were divided to three subgroups of aggressiveness (low aggressiveness, intermediate aggressiveness, high aggressiveness) using clinical criteria as described in “Materials and Methods”. The demographic variables were compared between normal research subjects and men with primary PCa or mCRPCa using one-way ANOVA and an extension of Fisher’s exact test for continuous and categorical variables, respectively. Age, PSA and Gleason scores were categorized as: 40-49, 50-59, 60-69, 70-79; 4, 4.1-10, 10.1; and 6, 7, 8, respectively.

Serum glutamate levels were compared for variables of interests and among the research subjects using one-way ANOVA, with Tukey adjusted post-hoc pair-wise comparisons. All serum samples measurements were log-transformed prior to statistical analysis. Simple linear regression models were used to assess the association between research subject’s serum glutamate and clinical status (normal, primary PCa with low-, intermediate, or –high aggressiveness score, mCRPCa) while adjusting for demographic covariates. Tukey adjusted pair-wise comparisons were made between clinical status of research subjects and their demographic strata when appropriate. The covariate adjusted models considered a single demographic covariate (with and without interactions) as well as multiple demographic covariates, as this information is not applicable or available for normal research subjects and mCRPCa patients. Only research subjects with primary PCa were considered when Gleason score and stage were included as covariates. All model assumptions were verified graphically using Quantile-Quantile (QQ) and residual plots.

Results

Serum glutamate levels in normal and prostate cancer research subjects

The research subjects’ race, Gleason scores, PSA values, age and clinical aggressiveness were described in Supplementary Table S1. Serum creatinine values were within the expected range and did not differ significantly among research subject subgroups. Serum PSA levels were found to be significantly ($P < 0.001$) different among normal individuals (0.556 ± 0.03), patients with primary PCa (10.38 ± 2.19), and patients with mCRPCa (323.74 ± 51.43). The average age was found to be significantly higher ($P < 0.001$) in research subjects with mCRPCa (70.2 ± 1.0) than in those with primary PCa (62.0 ± 0.6) or

normal research subjects (61.4 ± 1.2). Serum glutamate levels were not significantly different among different age groups of normal research subjects (Supplementary Table S2, $P = 0.1735$).

The entire study cohort was analyzed regardless of age and race. When the normal research subjects ($n = 60$) used for comparison, serum glutamate levels significantly increased in men with primary PCa ($n = 197$, $P = 0.003$), but returned to normal levels in men with mCRPCa ($n = 109$, $P = 0.002$) (Fig. 1A, left). When the primary PCa patients were divided into low, intermediate, and high aggressiveness, glutamate levels were significantly different: (a) between normal research subjects and intermediate ($P < 0.001$) or high aggressiveness ($P < 0.001$); (b) between low aggressiveness group and intermediate ($P < 0.002$) or high aggressiveness ($P < 0.001$) groups; (c) between intermediate aggressiveness group and mCRPCa ($P < 0.001$), and (d) between high aggressiveness and mCRPCa ($P < 0.001$) (Fig. 1A, middle). Pairwise comparisons indicated a significant association between glutamate levels and Gleason score ($P < 0.001$). Glutamate levels differed significantly between Gleason 6 ($n = 81$) and Gleason 7 ($n = 72$; $P < 0.001$) or Gleason 8 ($n = 30$, $P < 0.001$). Glutamate levels were higher in Gleason 8 than the Gleason 7 groups, but the difference did not reach statistical significance ($P < 0.064$) (Fig. 1A, right). Univariate analyses, after adjusting for PSA or stage, revealed no significant association between serum glutamate levels and any of the three aggressiveness categories in the pool of research subjects with primary PCa (Table 1).

The association between PCa aggressiveness and serum glutamate were explored among primary PCa research subjects while adjusting for potential confounders. Age, PSA, and stage did not appear to be confounders in the single and two covariate (confounder) models; they were non-significant and did not change the direction or significance of the relationship between serum glutamate levels and PCa aggressiveness. However, the inclusion of Gleason score appeared to over-power the relationship between serum glutamate levels and PCa aggressiveness. The Gleason score appeared to drive the difference between serum levels within the primary PCa research subjects (Table 1 and 2).

Serum glutamate levels in Caucasian Americans versus African Americans

The adjusted analysis involving race indicated that patient clinical status (primary or mCRPCa; $P < 0.001$), race ($P = 0.029$) and interaction ($P = 0.029$) effects were significant (Table 1). The pair-wise comparisons of race within each clinical subgroup of research subjects indicated that a significant difference exists in patients with mCRPCa ($P < 0.037$). Figure S1 demonstrates that this difference is the result of the significant interaction term, where serum glutamate decreases for Caucasians from the high aggressiveness to mCRPCa, but increase for African Americans.

Among Caucasian Americans, pair-wise comparisons indicated that significant differences in serum glutamate existed between research subjects with primary PCa and mCRPCa ($P < 0.030$) (Fig. 1B, left), between normal research subjects and those with PCa of high aggressiveness ($P < 0.004$), between low and high aggressiveness subgroups ($P = 0.001$), and between mCRPCa and high aggressiveness subgroups ($P < 0.001$) (Fig. 1B, middle). Among Caucasian American research subjects with primary PCa, a significant difference was found between research subjects with Gleason score 6 and Gleason score 7 ($P < 0.002$) and between Gleason score 6 and Gleason score 8 ($P < 0.001$) (Fig. 1B, right).

Among African Americans, pair-wise comparisons indicated that serum glutamate levels differed between normal research subjects and primary PCa ($P < 0.026$) and mCRPCa ($P < 0.016$) (Fig. 1C, left). A significant difference in serum glutamate was found in African Americans between the normal research subjects and intermediate aggressiveness subgroup

($P < 0.014$) and between normal and both high aggressiveness subgroup ($P < 0.027$) and mCRPCa ($P < 0.038$) patients (Fig. 1C, middle). Among African-American research subjects with primary PCa, significant differences in serum glutamate levels were found between research subjects with Gleason score 6 or Gleason score 8 ($P < 0.02$) (Fig. 1C, right). The serum glutamate levels differed significantly between the races only in the research subjects with mCRPCa ($P = 0.037$; Supplementary Fig. S1).

Immunohistochemical staining of metabotropic glutamate receptor 1 (GRM1) in benign and malignant prostate tissues

Specificity of anti-GRM1 immuno-reactivity was demonstrated by deleting the primary antibody or replacing it with a non-immune serum (data not shown). Basal cells exhibited nuclear staining whereas no staining was found in luminal acinar epithelial cells of BPH or normal prostate ($n = 10$; Fig. 2A). Endothelial cells, infiltrating macrophages, and stromal cells exhibited intense nuclear staining (Fig. 2A, mesenchymal cells). Intense cytoplasmic and nuclear staining was observed in proliferating epithelial cells of glands with basal cell hyperplasia (Supplementary Fig. S2). Moderate to intense cytoplasmic staining was noted in high-grade prostate intraepithelial neoplasia (HGPIN) (Fig. 2A). To further determine the GRM1 expression during PCa progression and metastasis, we carried out additional IHC staining in primary ($n = 15$) and metastatic PCa tissues ($n = 6$). Moderate to intense cytoplasmic staining with perinuclear enhancement was noted in Gleason score 6 and 7 PCa (Fig. 2A). Intense cytoplasmic staining with perinuclear enhancement was also observed in malignant acinar cells of Gleason score 8 (4+4), 9, and 10 (Fig. 2A) tumors. PCa cells invading into extraprostatic adipose and muscular tissues exhibited intense cytoplasmic staining (Fig. 2A). Intense nuclear staining was observed at tumor-inflammatory interfaces in inflammatory cells, endothelial cells, and isolated macrophages (Fig. 2A). Intense cytoplasmic staining was found in multi-layer PCa cells infiltrating around nerves (Fig. 2A). Intense cytoplasmic staining with perinuclear enhancement was detected in PCa cells metastatic to bone and the abdominal wall (Fig. 2A and Supplementary Fig. S3). GRM1 staining appeared to be distributed in both nuclear and cytoplasmic compartments. The nuclear staining of GRM1 was heterogeneous.

GRM1 expression in prostate cancer cell lines

An affinity purified anti-GRM1 antibody detected a strong band at ~ 85 kDa (Fig. 2B) and three other weakly expressing bands at ~ 40, ~60, and ~ 170 KDa, which were close to the predicted bands based on GRM1 molecular weight described by the manufacturer. Parallel immunoblotting with anti-GRM1 antibody pre-absorbed by its immunogenic peptide confirmed specificity (Fig. 2B).

GRM1 expression in androgen-sensitive LNCaP cells was less than androgen-independent PC-3, DU145 and androgen-independent and bone metastatic PCa cell lines (i.e., MDA-PCa 2b and VCaP). GRM1 expression in the E006AA cell line obtained from an organ-confined Gleason 6 score PCa from an African-American was less than bone-metastatic MDA-PCa2b cells (Fig. 2B). GRM1 expression in 22Rv1 cells was higher than LNCaP cells.

Prostate cancer cells growth in glutamate-free culture condition

Proliferation of PC-3 cells was decreased by 42%, DU145 cells by 63%, and LNCaP cells by 68%, as compared to their isogenic control cell types after culturing 3, 5, or 7 days in customized glutamine- and glutamate-free medium supplemented with dialyzed FBS and GlutaMAX (Fig. 3A).

Inhibition of PCa cell proliferation by GRM1 antagonists

To evaluate the potential of intracellular glutamate levels on PCa cells growth, we used riluzole which functions in part as the glutamate release inhibitor (14, 15). Inhibition of glutamate release in a cell type-, dose-, and time-dependent manner decreased PCa cells growth (Fig. 3B). LNCaP cells were the most sensitive among the PCa cell lines investigated. LNCaP cells growth was reduced by 54% (day 2) and by 85% (day 6) compared with their control cell type at the same incubation period (Fig. 3B, right). Among all PCa cells, DU145 cells were least sensitive to riluzole (Fig. 3B, middle). Proliferation of DU145 cells was decreased by 14% (day 2) and by 18% (day 6). However, PC-3 growth was decreased by 10% (day 2) and by 40% on day 6 (Fig. 3B, left).

To demonstrate GRM1 responsiveness as a functional glutamate receptor, independent studies were performed using BAY36-7620 as a non-competitive GRM1 antagonist at different concentrations and for a period of 2 to 6 days. As in the riluzole study, cells were maintained in their complete medium containing non-dialyzed FBS and L-glutamine (2mM). BAY36-7620 showed similar growth inhibitory effect on the three PCa cell lines and LNCaP cells were found to be the most sensitive cell line (Supplementary Fig. S4).

Glutamate blockade induces apoptotic cell-death

The expression of cleaved (active) forms of the initiator caspase-9 and its active downstream effectors (caspases-7 and -3) were determined in the cells grown in the presence of riluzole for 4 or 6 days. The expression of cleaved caspase-9, -7, and -3 increased in a cell type-specific and dose-dependent manner in all cells investigated (Fig. 3C). In general, riluzole induced a cell type-specific increase in the expression level of initiator and effector caspases.

The intensity of cleaved-PARP expression increased in a dose-dependent manner in all PCa cell lines. H2AX, a member of H2A histone family is phosphorylated on serine residue 139 (known as γ -H2AX) after the activation of the caspase cascade and as a result of apoptotic DNA fragmentation (26). Cell lysates showed a dose-dependent increase in phospho-H2AX^{Ser139} levels in LNCaP cells (Fig. 3C). An increase in γ -H2AX levels was also detected at 50 μ M riluzole in PC-3 and DU145 cells. Accumulation of intracellular glutamate levels secondary to riluzole treatment appeared to be apoptogenic.

Glutamate blockade decreases PCa cells migration and invasion

Addition of riluzole or BAY36-7620 to both upper and lower compartments of transwell filters decreased cell motility and invasion in a dose-dependent manner in PC-3 and DU145 cells (Fig. 4A and B). Riluzole reduced migration of PC-3 cells by 53% and DU145 cells by 76% and invasion of PC-3 by 65% and DU145 by 82% (Fig. 4A and B). BAY36-7620 decreased migration of PC-3 cells by 67%, DU145 cells by 88% and invasion of PC-3 by 83%, and DU145 by 96% (Fig. 4A). Similar data was obtained using a wound assay (Supplementary Fig. S5A-C).

Discussion

Serum glutamate levels positively correlated with Gleason score and PCa aggressiveness. IHC staining showed weak or no expression of GRM1 in luminal acinar cells of normal or benign glands, but high expression levels in primary and metastatic PCa tissues. Glutamate deprivation or blockade with GRM1 antagonists decreased PCa cells growth, migration, and invasion and led to apoptotic cell death.

Glycolysis and glutaminolysis are major contributing metabolic pathways of tumorigenesis. Glutamine is the most abundant serum amino acid and a necessary metabolic precursor for other amino acids and nucleotides. Glutamine deprivation decreased cell proliferation, DNA synthesis, and protein synthesis rates in a cell type-dependent manner in hepatocellular carcinoma and several breast and colon adenocarcinoma cells (27). Similarly, the importance of glutamate signaling in cancer has been revealed by antiproliferative activity of glutamate antagonists in different types of human tumor cells, such as melanoma, colon adenocarcinoma, breast carcinoma, astrocytoma, and lung carcinoma (17).

Humoral response profiling of serum samples by Taylor *et al*/revealed elevated levels of nitrogen metabolism during PCa progression (10). Concomitant validation of increased levels of the constituents of nitrogen metabolism pathway was determined further by using high throughput quantitative profiling of relative levels of metabolites in BPH (n = 16) and organ-confined PCa (n = 12) tissues. This study revealed significantly increased levels of glutamate in the PCa tissues ($P = 0.0003$) compared to BPH. Further examination of metabolomic changes during PCa progression by the same group verified elevated levels of glutamate in a majority of PCa tissues as compared with benign glands (11).

The excess amount of glutamate in PCa tissues and patients' serum may be explained by: (a) increased rate of glutaminolysis or glutamine addition in proliferating PCa cells and (b) carboxypeptidase function of prostate-specific membrane antigen (PSMA) overexpressed by PCa cells. While "glutamine addiction" serves as a non-specific metabolic hallmark of malignant and proliferating cells, it is likely that the known enzymatic activity of PSMA may have a key contributing role in glutamate production by PCa cells. PSMA is a type II transmembrane glutamate carboxypeptidase and glutamate is the major constant product of its enzymatic activity in PCa cells (28, 29). PSMA is overexpressed in PCa and high PSMA expression is associated with advanced tumor stage, poor prognosis and high risk of biochemical recurrence (28,30). PSMA-generated glutamate signaling may serve as a non-androgenic regulator of PCa growth (31). Therefore, PSMA may have critical functions in metabolomics profiling during prostate carcinogenesis and progression.

Analysis of the glutamate receptor, GRM1, in PCa cell lines showed a cell type-dependent expression. GRM1 expression was higher in metastatic or androgen-independent PCa cell lines (e.g., DU145, MDA-PCa2b, VCaP, 22RV1) than in the primary (i.e., E006AA) or androgen-sensitive LNCaP cell lines. GRM1 overexpression might be an adoptive change hypersensitizing PCa cells to extracellularly available glutamate or could confer oncogenic activity due to its post-receptor signaling activation. Aberrant expression of GRM1 was found sufficient for oncogenic transformation of melanocytes in transgenic mice (18, 19). Overexpression of several members of mGluRs have been reported in other cancers (32-34). Activation of mGluR-triggered signaling pathways may occur in response to free circulating glutamate, glutamate released by tumor cells (autocrine loop), or stromal cells in the tumor microenvironment (paracrine loop). The data presented here also support an intracrine regulatory role for glutamate in PCa cell growth, migration, and invasion.

Intense nuclear staining was detected in basal cells of normal glands and in endothelial cells, infiltrating macrophages, and stromal cells at the tumor-inflammatory interface or tumor microenvironment. Moderate to intense cytoplasmic staining was detected in HGPIN, which is considered to be a precursor lesion to PCa. Further examination of GRM1 expression in primary and metastatic PCa tissue sections showed moderate to intense cytoplasmic staining in malignant acinar cells with perinuclear enhancement in Gleason score 7 tumors and intense but similar expression pattern in Gleason score 8 tumors and in highly aggressive ductal adenocarcinomas (Supplementary Fig. S3). In addition, locally advanced PCa produced intense cytoplasmic staining in PCa cells invading into extraprostatic adipose and

muscular tissues, and in multi-layer tumor cells infiltrating around nerve cells. Intense cytoplasmic staining with perinuclear enhancement was detected in metastatic PCa cells to bone and the abdominal wall. Overall, the descriptive IHC analysis demonstrates GRM1 overexpression in primary and metastatic PCa tissues compared to non-cancerous and benign tissues. Additional large-scale studies are required to assess the predictive or prognostic significance of GRM1 expression in PCa.

Serum glutamate levels in mCRPCa were significantly higher in African-American patients than Caucasian-American research subjects. Univariate analysis showed serum glutamate levels affected by race (Table 1). Two covariate models adjusting for two potential confounders revealed that age, PSA, race, and stage are not significant confounders (Table 2). However, when Gleason score was added to the model with race, age, and PSA, race had a dominant effect and relationship with serum glutamate levels in African-American men. Gleason score is the most reliable predictor of lethal PCa. Both groups showed significant association between serum glutamate levels and Gleason score (6 versus 8). African Americans with mCRPCa were found to have higher serum glutamate levels than in the primary PCa. These data might reflect the small sample size for mCRPCa (n= 10) in African American study cohort compared with the Caucasian Americans (n=99).

These data suggest that high serum glutamate levels may favor higher growth rates and aggressive behavior for primary PCa in both racial groups and for mCRPCa in African Americans. While inter-racial differences for metabolic genes have been reported in several studies (35), large-scale studies are needed to determine the exact contribution of glutamate either as a metabolic byproduct or a contributing factor in PCa-racial disparity.

First evidence for a link between glutamate receptor mGluR1 (GRM1) and tumorigenesis was provided by an accidental discovery in which spontaneous melanoma in a transgenic mouse line (TG-3) was discovered to be due to multiple tandem insertions of the transgene into intron 3 of *GRM1* leading to aberrant expression of GRM1 in melanocyte and the development of melanoma at a high rate and young age (18, 19). This was verified in a subsequent transgenic mouse line, TG(*GRM1*)E_{pv} (E), in which *GRM1* expression was regulated by a melanocyte-specific promoter, dopachrome tautomerase (36). Different pharmacologic agents have been used as GRM1 (i.e., mGluR1) agonist or antagonists. Riluzole is an FDA-approved GRM1-blocking agent and well-tolerated oral medicine for the treatment of amyotrophic lateral sclerosis that has been used to prevent glutamate release (37). LY367385 or BAY36-7620 is known as competitive or non-competitive GRM1 antagonist, respectively (24, 38). Riluzole, BAY36-7620, or LY367385 prevent the growth promoting effect and activation of signaling pathways by glutamate in a variety of cancer cell types including melanoma and breast carcinoma (38, 39).

PCa cell proliferation was significantly reduced in a time-dependent manner in glutamate-depleted medium or in the presence of GRM1 antagonists (Fig. 3A and B and Supplementary Fig. S4). These data indicated that PCa cells growth depended on GRM1-signaling. Riluzole inhibition of glutamate release and GRM1-signaling in PCa cells cultured in complete growth medium not only decreased growth rate, but led to apoptotic cell death, demonstrated by increased expression levels of cleaved caspase-9, -7, -and -3 and PARP. These results were confirmed by the demonstration that phospho-H2AX^{Ser139} levels (known as γ -H2AX) appeared early during apoptosis. Initiation of DNA double-strand breaks following caspase and apoptotic endonuclease activation induces phosphorylation of H2AX histone at Ser139 (26).

The inhibitory effect of glutamate blockade on PCa cell proliferation and migration may be at least partially mediated by the PI3K/Akt/mTOR pathway (6). The PI3K/Akt/mTOR

function as the main regulator of aerobic glycolysis in malignant cells. Activation of the PI3K/Akt/mTOR pathway in cancer cells triggers many of the metabolic activities that support cellular biosynthesis such as (a) increased surface expression of nutrient transporters leading to increased uptake of glucose and amino acid, (b) activated Akt increases glycolysis and lactate production and induces a “Warburg effect” in cancer or even proliferating non-transformed cells (40), (c) activation of PI3K and Akt induce lipogenesis and stimulate lipogenic genes expression in a variety of cell types (41), and (d) mTOR serves as a major regulator of protein translation.

A cell type-specific response to glutamate blockade suggests differential expression levels for GRM1 in different PCa cells or their dependence on glutamate signaling. Excess intracellular glutamate and/or inhibition of GRM1-signaling by riluzole treatment may have deleterious effects on PCa cells growth, migration, and invasion which cannot be salvaged by an intact extracellularly-activated GRM1-signaling. In addition, accessibility to a certain amount of glutamate is essential for PCa cells growth that may signify “glutamine addiction”. However, more than this necessary amount may lead to “glutamate-toxicity” and activation of various interactive apoptotic signaling pathway leading to PCa cell-killing. Intracellular glutamate as a metabolic intermediate, metabolic byproduct, or metabolic regulator of interactive multiple signaling pathways may play a major role in malignancy. Glutamate via a combination of intracrine, autocrine, paracrine pathways may contribute to PCa growth and aggressiveness. Our data suggest that glutamate together with other predictive or prognostic factors may be a useful metabolic marker for early detection and clinical discrimination of aggressive tumors from non-aggressive tumors. Glutamate-initiated signaling pathways may provide novel therapeutic opportunities for PCa. Additional work is needed to ascertain the clinical and therapeutic implications of glutamate dependence in PCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Aerobic glycolysis is a hallmark of proliferating normal and malignant cells. During glutaminolysis, glutamine is catabolized to glutamate and incorporated into the citric acid cycle and lipogenesis. Glutamate plays a substantial and overlapping role between metabolic and oncogenic signaling pathways. In this study, we found that serum glutamate levels were increased in primary PCa patients and directly correlated with Gleason score and PCa aggressiveness. Compared with Caucasian Americans, serum glutamate levels were higher in African Americans with mCRPCa than those with primary tumors. Immunohistochemical staining showed weak or no expression of metabotropic glutamate receptor 1 (GRM1) in normal prostate cells, but overexpression in primary and metastatic PCa. Glutamate-deprivation or blockade with GRM1 antagonists reduced PCa cells' growth, migration, and invasion and proved apoptogenic. Glutamate may provide a metabolic biomarker of PCa aggressiveness and glutamine metabolism or signaling may provide a target for therapeutic intervention.

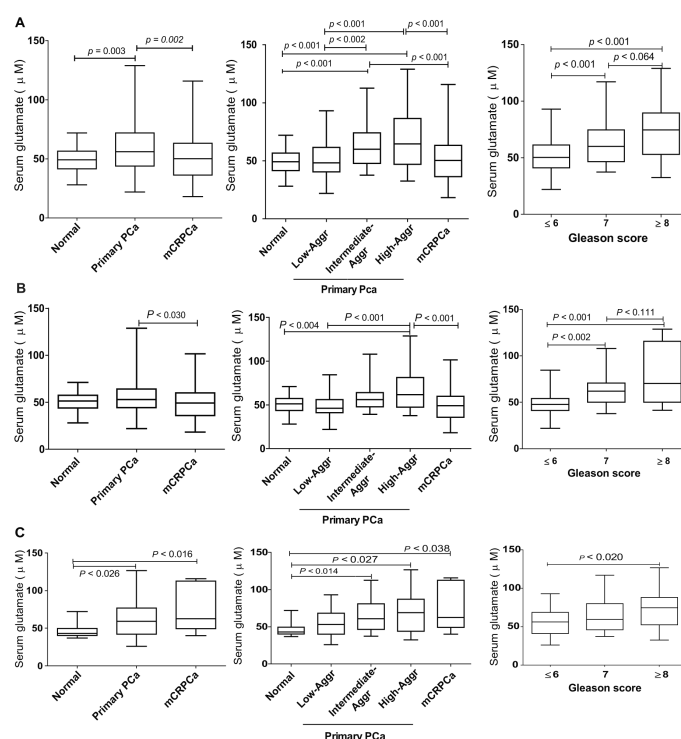


Figure 1. Box and whiskers plot of serum glutamate levels in normal individuals and patients with primary or metastatic castrate-resistant PCa of Caucasian- or African-American origin Serum glutamate levels were measured as described in “Materials and Methods”. The study cohorts were analyzed according to their clinical status as normal research subjects, primary PCa, or metastatic castrate-resistant PCa (left). All primary PCa patients were divided according to their aggressiveness score based on Gleason and PSA (middle) or according to Gleason score (right). **A**, The entire study cohorts. **B**, The entire study cohorts of Caucasian Americans. **C**, The entire study cohorts of African Americans. Actual *p*-values are indicated on individual graphs. The distribution of log-transformed serum measurements were compared among groups using one-way ANOVA with Tukey pair-wise comparisons. The box frame defines the 25-75 percentiles, the ends of error-bar like whiskers depict the minimum and maximum values, and the line within the box marks the median value.

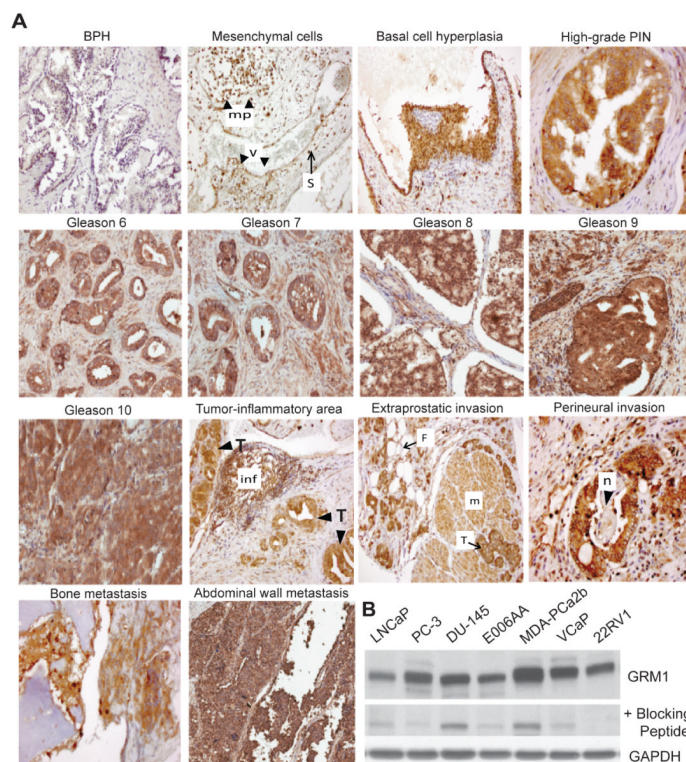


Figure 2. Expression pattern of metabotropic glutamate receptor 1 (GRM1) in normal and malignant prostate cancer tissues and cells

A, Representative immunohistochemistry results; magnification, x 200. Benign prostate hyperplasia showing nuclear staining in basal cells and absence of staining in normal luminal acinar cells. Intense nuclear and cytoplasmic staining is noted in vascular endothelial cells (v), tissue infiltrating macrophages (mp), and stromal cells (s). Intense cytoplasmic and nuclear staining is seen in an area of basal cell hyperplasia. Moderate to intense cytoplasmic staining is noted in a representative image of high-grade prostate intraepithelial neoplasia (HGPIN), with relatively absence of staining in most nuclei. Moderate to intense cytoplasmic staining with perinuclear enhancement is noted in Gleason 6 (3+3) and Gleason 7 (3+4) tumor. Intense cytoplasmic staining with perinuclear enhancement was observed in malignant luminal acinar cells with Gleason 8 (4+4), Gleason 9 (5+4), and a Gleason 10 tumor. Moderate cytoplasmic staining in a Gleason 3+4 tumor with intense nuclear staining was observed in inflammatory (inf) and endothelial cells, and isolated macrophage. Intense cytoplasmic staining was observed in tumor cells invading into extraprostatic adipose (F) and muscle (M) tissues. Perineural invasion was noted by intense cytoplasmic staining of multi-layer tumor cells circling around nerve cells (n). Neural structures are negative for staining and the background stroma shows moderate focal nuclear staining. Intense cytoplasmic staining with perinuclear enhancement is noted in scattered bone-metastatic prostate cancer cells. Bone tissue appears as fragmented pieces. Intense cytoplasmic staining was observed in malignant prostate cells metastasized into abdominal wall. **B**, GRM1 protein expression in prostate cancer cell lines. Western blot analysis was performed on whole-cell lysates by SDS-PAGE in the presence of reducing agent (upper panel). The anti-GRM1 specificity was examined by probing a duplicate membrane with primary antibody pre-incubated with 5 µg/ml blocking peptide for 2h (middle panel). GAPDH detection was used for loading control. LNCaP = androgen-sensitive PCa cell line; PC-3, DU145, MDA-PCa2b, VCaP, 22RV1 androgen-independent PCa cell lines; E066AA = primary African American PCa cell line.

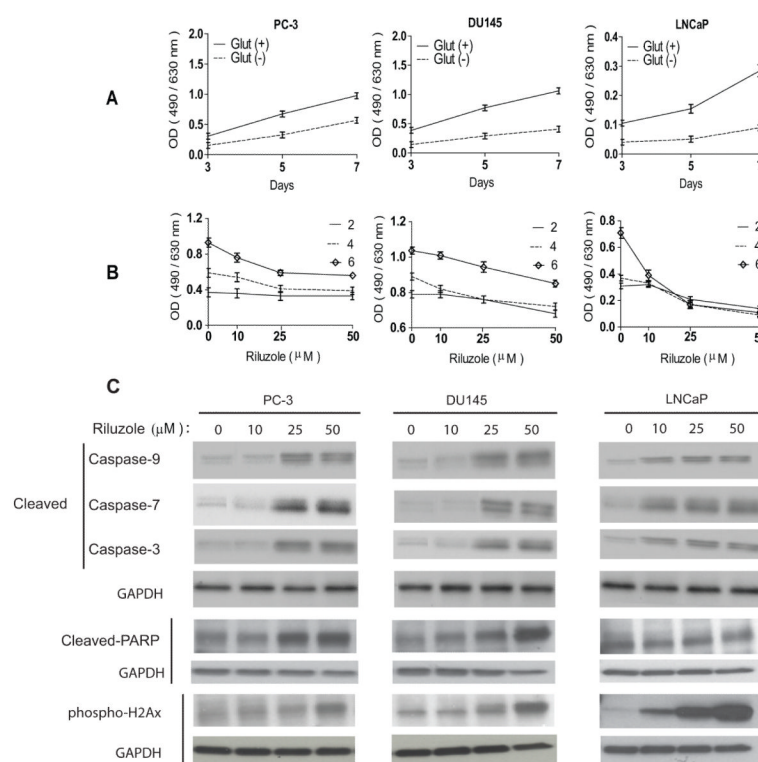


Figure 3. Inhibition of prostate cancer cell growth by glutamate deprivation or GRM1-antagonist

A, Effect of glutamate deprivation on PCa cells proliferation. Cells were seeded at 500 (PC-3, DU145, LNCaP) per 200 μ l/well in 24 replicates in 96-well plates in their complete medium. After 3 days, cells were incubated in their maintenance medium or customized glutamine- and glutamate-free culture media supplemented with 10% dialyzed FBS and 2 mM GlutamaxTM for 3, 5, or 7 days. The media were refreshed every 48 h. Cell proliferation was measured by adding 20 μ l MTS solution per well for 1 h and measuring the absorbance at 490 / 630 nm. bars, mean \pm SEM, $p < 0.001$, Student t -tests were used to compare cell growth between glutamate-free and complete medium at each day. One-way ANOVA test with Bonferroni corrections was used to compare values among different days for each cell line. **B**, Effect of GRM1-antagonist on PCa cells growth. Cells were seeded as described above and were incubated in their complete medium in the presence or absence of riluzole at 10, 25, or 50 μ M for 2, 4, or 6 days with media refreshment every 48h, followed by cell proliferation assay as described above. Data represented the average of three independent experiments \pm SEM. Statistical significance ($p < 0.0001$) between the control and treatment groups was evaluated by one-way ANOVA test with Bonferroni adjustment. **C**, Induction of apoptosis by riluzole. Cells were cultured in their complete medium in the presence or absence of riluzole as described above. Whole cell lysate were extracted and immunoblotting was performed on 30 μ g protein using antibodies against cleaved caspases-9, -7, and -3, PARP, and phosphoserine-139 H2AX. Anti-GAPDH antibody was used for control loading. Experiment was repeated twice independently.

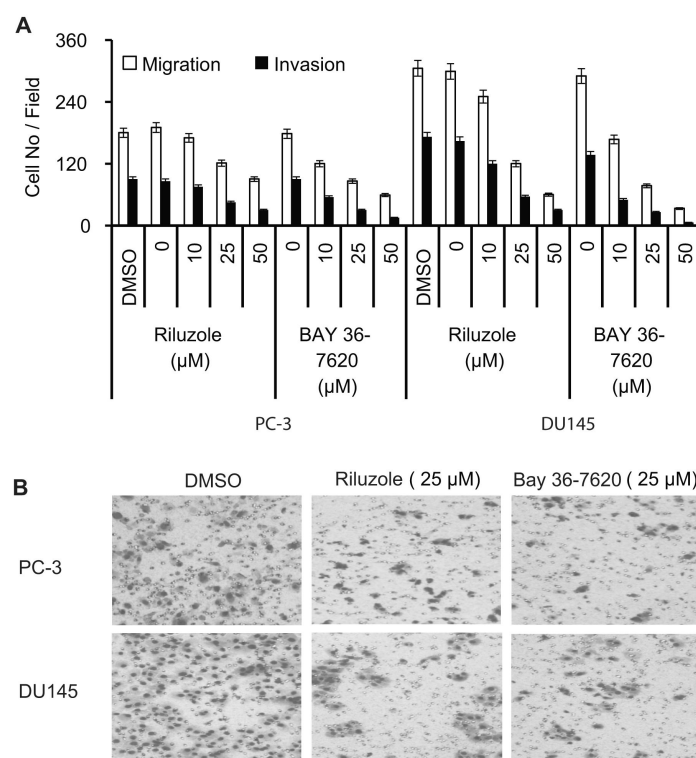


Figure 4. Effect of glutamate blockade on PCa cells migration and invasion

Cell migration and invasion was performed using 8-μm transwell filters. For the invasion assay, the upper compartment was coated with 50 μg Matrigel per filter. A suspension of cells (5×10^4 for PC-3 or 2×10^4 for DU145) in basal medium containing 0.1% BSA were added to the upper compartment. The lower compartment was filled with 400 μl basal medium containing 10% FBS as chemoattractant. Riluzole or BAY36-7620 was included at equimolar concentrations (0 to 50 μM) to upper and lower compartments. After 48 h for PC-3 or 24 h for DU145, migrated or invaded cells in transwell filters were counted from ten randomly selected fields in at least four independent wells. Data represented the average of three independent experiments \pm SEM. Statistical significance ($p < 0.05$) between the control and treatment groups was evaluated by one-way ANOVA test with Bonferroni adjustment.

Table 1

Single covariate adjusted analysis of serum glutamate levels in normal individuals, patients with primary or metastatic castrate-resistant prostate cancer

Variable	Normal Glu ($\mu\text{M} \pm \text{SE}$) (n = 60)	Primary PCa (Aggressiveness subgroup) Glu ($\mu\text{M} \pm \text{SE}$)			mCRPCa Glu ($\mu\text{M} \pm \text{SE}$) (n = 109)	P-Value ^a
		Low (n = 80)	Intermediate (n = 68)	High (n = 49)		
Gleason Score^{b,c} 6 (n = 94) 7 (n = 73) 8 (n = 30)	N/A ^e	51.7 \pm 1.8 (n=80) N/A ^f (n=0) N/A ^f (n=0)	53.8 \pm 3.5 (n=13) 65.8 \pm 2.8 (n=55) N/A ^f (n=0)	86.0 \pm N/A ^d (n=1) 55.9 \pm 4.8 (n=18) 75.7 \pm 5.0 (n=30)	N/A ^e	Aggressiveness subgroup ^g : 0.222 Gleason: 0.007
PSA^b (ng/ml) 4 (n = 119) 4.1-10 (n = 100) 10.1 (n = 139)	49.3 \pm 1.4 (n=60) N/A ^e N/A ^e	51.3 \pm 2.4 (n=39) 52.6 \pm 2.5 (n=41) N/A ^f (n=0)	68.0 \pm 6.4 (n=11) 63.6 \pm 3.7 (n=37) 61.8 \pm 4.0 (n=20)	86.1 \pm 12.0 (n=6) 57.5 \pm 5.6 (n=16) 68.0 \pm 4.5 (n=27)	60.9 \pm 11.5 (n=3) 69.9 \pm 14.5 (n=6) 50.9 \pm 1.8 (n=92)	Clinical status ^h : <0.001 PSA: 0.252 Int: 0.178
Stage^b T1 (n = 47) T2 (n = 113) T3 (n = 37)	N/A ^e	57.2 \pm 3.7 (n=26) 49.5 \pm 1.9 (n=54) N/A ^f (n=0)	69.4 \pm 5.9 (n=14) 63.3 \pm 2.9 (n=44) 56.1 \pm 6.2 (n=10)	73.9 \pm 10.5 (n=7) 64.9 \pm 7.1 (n=15) 69.4 \pm 5.1 (n=27)	N/A ^e	Aggressiveness subgroup ^g : <0.001 Stage: 0.179 Int: 0.591
Race Whites (n = 222) Blacks (n = 144)	50.3 \pm 1.7 (n=44) 46.8 \pm 2.3 (n=16)	48.8 \pm 2.4 (n= 35) 54.4 \pm 2.5 (n=45)	58.5 \pm 3.0 (n=25) 66.4 \pm 3.3 (n=43)	70.7 \pm 6.5 (n=19) 67.4 \pm 4.7 (n=30)	50.6 \pm 1.7 (n=99) 72.6 \pm 9.4 (n=10)	Clinical status ^h : <0.001 Race: 0.029 Int: 0.029

^aIn each scenario a linear regression model was used to evaluate the association between patient clinical status and log-transformed serum glutamate while adjusting for different patient characteristics and their interaction. All model assumptions were checked graphically using Quartile-Quartile and residual-plots. Data are presented as mean \pm SE.

^bOnly Primary PCa patients were included in the analysis. PSA data was not available for all research subjects in the mCRPCa subgroup.

^cInteraction term not considered given the frequency of sparse cell counts.

^dCannot compute the SE based on a single observation. PSA value for this research subject was 28.0 ng/ml.

^eThe variable of interest was not applicable or analyzed for the specified research subjects.

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^fNo research subject was identified in the low-aggressiveness subgroup for the variable of interest.

^gComparison made among aggressiveness subgroups of primary PCa patients

^hClinical status refers to comparison made among all normal research subjects, aggressiveness subgroups of primary PCa patients, and mCRPCa patients
Abbreviations: Glu, glutamate; mCRPCa, metastatic castrate-resistant PCa; N/A, not available or not applicable; SE, standard error; Int, interaction term; Med, medium; PSA, prostate-specific antigen.

Table 2

Multiple covariate adjusted analysis of serum glutamate levels in patients with primary and metastatic castrate-resistant prostate cancer

Adjusted Variable Subtype	Variable of interest	Primary PCa (Aggressiveness Subtype) Glu ($\mu\text{M} \pm \text{SE}$)			mCRPCa Glu ($\mu\text{M} \pm \text{SE}$)	P-values ^a
		Low	Intermediate	High		
Race Whites Blacks	Gleason Score 6 7	48.2 \pm 2.4 71.5 \pm n/a ^b	47.9 \pm 1.8 63.4 \pm 3.7 N/A	N/A 56.2 \pm 5.2 79.1 \pm 9.1	N/A	Aggressiveness subgroup ^d : 0.216 Race: 0.201 Gleason: 0.008
	White	54.4 \pm 2.5	63.3 \pm 7.2	86.0 \pm n/a ^b		
	Black	N/A	66.8 \pm 3.7	55.7 \pm 7.4		
	White	N/A	N/A	73.4 \pm 5.9		
PSA (ng/ml) 4.0 4.1-10 10.1	Race White Blacks White Black White Black	47.9 \pm 2.4 55.4 \pm 4.4 49.7 \pm 4.2 54.4 \pm 3.2 N/A 42.2 \pm n/a ^b	60.6 \pm 4.3 76.7 \pm 12.5 62.3 \pm 6.2 64.2 \pm 4.6 54.3 \pm 3.6 65.6 \pm 5.5	86.9 \pm 12.8 82.8 \pm 43.8 55.3 \pm 6.4 58.5 \pm 7.8 64.8 \pm 4.1 69.1 \pm 5.9	60.9 \pm 11.5 N/A 60.9 \pm 14.0 114.5 \pm n/a ^b 49.1 \pm 1.7 68.0 \pm 9.2	Clinical status ^c : <0.001 PSA: 0.094 Race: 0.034
	White					
	Black					
	Black					
Age (y) 40-49 50-59 60-69 70+	Gleason Score 6 7	58.3 \pm 5.6 N/A N/A	59.9 \pm 0.8 71.9 \pm 15.4 N/A	N/A 48.1 \pm 0.1 59.3 \pm n/a ^b	N/A	Aggressiveness subgroup ^d : 0.175 Age: 0.419 Gleason: 0.005
	White	51.0 \pm 3.0	46.7 \pm 7.5	86.0 \pm n/a ^b		
	Black	N/A	71.2 \pm 5.4	70.1 \pm 14.8		
	Black	N/A	N/A	79.5 \pm 10.5		
PSA (ng/ml) 4.0 4.1-10 10.1	Gleason Score 6 7	50.9 \pm 2.8 N/A N/A	49.9 \pm 1.0 64.1 \pm 3.7 N/A	N/A 52.6 \pm 4.1 77.3 \pm 7.9	N/A	Aggressiveness subgroup ^d : 0.247 PSA: 0.502 Gleason: 0.020
	White	51.8 \pm 5.1	56.1 \pm 7.3	N/A		
	Black	71.5 \pm n/a ^b	61.1 \pm 6.6	43.0 \pm 1.9		
	Black	N/A	N/A	71.9 \pm 9.7		
PSA (ng/ml) 4.0 4.1-10 10.1	Gleason Score 6 7	50.6 \pm 2.4 71.5 \pm n/a ^b	N/A 68.0 \pm 6.4 N/A	N/A 44.9 \pm n/a ^b 90.6 \pm 12.4	N/A	Aggressiveness subgroup ^d : 0.247 PSA: 0.502 Gleason: 0.020
	White	52.6 \pm 2.5	48.5 \pm 2.5	N/A		
	Black	N/A	66.3 \pm 4.1	57.2 \pm 4.4		
	Black	N/A	N/A	57.7 \pm 9.0		
PSA (ng/ml) 4.0 4.1-10 10.1	Gleason Score 6 7	42.2 \pm n/a ^b N/A	57.1 \pm 5.3 65.6 \pm 5.8 N/A	86.0 \pm n/a ^b 58.0 \pm 7.6 76.3 \pm 3.8	N/A	Aggressiveness subgroup ^d : 0.247 PSA: 0.502 Gleason: 0.020
	White					
	Black					
	Black					

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^aIn each scenario a linear regression model was used to evaluate the association between aggressiveness subgroups of research subjects with primary PCa or clinical status of the research subject and log serum glutamate while adjusting for different combinations of patient characteristics. All model assumptions were checked graphically using Quartile-Quartile and residual-plots.

^bCannot compute the SE based on a single observation

^cClinical status refers to comparison made among all normal research subjects, aggressiveness subgroups of primary PCa patients, and mCRPCa patients

^dComparison made among aggressiveness subgroups of primary PCa patients

Abbreviations: Glu, glutamate; mCRPCa, metastatic castrate-resistant PCa; N/A, not available or not applicable; SE, standard error; Int, interaction term; Med, medium; PSA, prostate-specific antigen.